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A heart mitochondrial Ca^{2+}-dependent pore of possible relevance to re-perfusion-induced injury

Evidence that ADP facilitates pore interconversion between the closed and open states

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The permeability properties of a putative Ca^{2+}-activated pore in heart mitochondria, of possible relevance to re-perfusion-induced injury, have been investigated by a pulsed-flow solute-entrapment technique. The relative permeabilities of [^{14}C]mannitol, [^{14}C]sucrose and arsenazo III are consistent with permeation via a pore of about 2.3 nm diameter. Ca^{2+} removal with EGTA induced pore closure, and the mitochondria became 'resealed'. The permeability of the unresealed mitochondria during resealing was markedly stimulated by 200 μM-ADP, and the relative permeabilities to solutes of different size were stimulated equally, indicating an increase in open-pore number, rather than an increase in pore dimensions. This is paradoxical, since ADP also stimulated the rate of resealing. The rate of EGTA-induced resealing was also stimulated by the Ca^{2+} ionophore A23187, which indicates that the rate of removal of matrix free Ca^{2+} is limiting for pore closure. An explanation for the paradox is suggested in which ADP facilitates pore interconversion between the closed and open states in permeabilized mitochondria, and pore closure in Ca^{2+}-free mitochondria occurs much faster than previously thought.

INTRODUCTION

Following the original observations of Rossi & Lehninger (1964) and Greenawalt *et al.* (1964), it is widely appreciated that excess Ca^{2+} can disrupt mitochondrial energy transduction (for a review, see Mela, 1977). It is also clear that the phenomenon involves non-specific permeabilization of the inner membrane to low-M_r solutes (Haworth & Hunter, 1980; Beatrice *et al.*, 1980; reviewed in Crompton, 1985*a*). The amounts of Ca^{2+} required are relatively high, the rate of permeabilization being far from saturated by 25 μM matrix free Ca^{2+} (Al Nasser & Crompton, 1986*a*); such concentrations greatly exceed those that control Ca^{2+}-sensitive matrix dehydrogenases (0.2–2 μM matrix free Ca^{2+}; Denton & McCormack, 1985; Hansford, 1985), and the adverse effects of Ca^{2+} are evidently well displaced from its physiological role in regulating citric acid-cycle activity. It has often been suggested that high [Ca^{2+}] may be detrimental to mitochondrial energy transduction in those forms of injury associated with excess cellular Ca^{2+}, notably that occurring during tissue anoxia/reoxygenation, and that mitochondrial dysfunction might contribute to the progression of the injury (Lochner *et al.*, 1975; Henry *et al.*, 1977; Chien *et al.*, 1977; Peng *et al.*, 1980; see also review by Crompton, 1988). As pointed out previously (Crompton *et al.*, 1988), there is a close correlation, in heart at least, between the level of resting cytosolic free Ca^{2+} above which irreversible injury occurs on reoxygenation (Allshire *et al.*, 1987) and the level at which mitochondrial Ca^{2+} overload would be predicted (Crompton, 1985*b*). It is important, therefore, to understand the nature of the lesion and the factors that influence it.

We have studied the reversibility of permeabilization as a means of gaining an insight into the underlying mechanism. These studies demonstrated that mere removal of Ca^{2+} induces complete reversal of permeabilization, i.e. resealing (Al Nasser & Crompton, 1986*a*,*b*). Resealing occurs within seconds of Ca^{2+} removal in the absence of ATP and other cofactors required for phospholipid reacylation; this, we have argued (Crompton & Costi, 1988), discriminates against a widely considered mechanism involving membrane leakiness and membrane repair brought about by deacylation (Ca^{2+}-activated phospholipase A_2) and reacylation of membrane phospholipids (Beatrice *et al.*, 1980; Broekemeier *et al.*, 1985; Riley & Pfeiffer, 1986). Following earlier suggestions by Haworth & Hunter (1980), we interpret these findings to indicate the presence of an inner membrane pore activated reversibly by Ca^{2+}. The possible involvement of pore-mediated mitochondrial dysfunction in reoxygenation-induced injury is strengthened by the fact that opening of the putative pore is promoted synergistically by Ca^{2+}, increased P_i and oxidative stress (Al Nasser & Crompton, 1986*a*; Crompton *et al.*, 1987, 1988; Crompton & Costi, 1988), all of which are recognized features of this form of injury (for reviews, see Poole-Wilson *et al.*, 1984; McCord, 1987).

In the present study the pulsed-flow solute-entrapment technique for the analysis of solute permeation (Crompton & Costi, 1988) is improved by linear plotting procedures. Application of these provides evidence for a dynamic distribution between closed and open states of the pore in permeabilized mitochondria and suggests that pore closure may occur considerably faster than previously thought.

METHODS

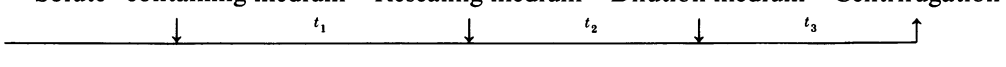
Mitochondrial preparation

Mitochondria were prepared from the hearts of female Sprague–Dawley rats (300–350 g body wt.) and their protein contents were determined as described previously (Crompton *et al.*, 1987).

Pulsed-flow measurements of solute permeation and pore closure

The rates of solute permeation into and out of permeabilized (open pore) mitochondria and the rates of resealing (pore closure) were measured by the pulsed-flow solute-entrapment technique described previously (Crompton & Costi, 1988). Heart mitochondria were first permeabilized by preincubation (at about 1 mg of protein/ml) in medium (pH 7.0) containing 120 mM-KCl, 10 mM-Hepes, rotenone (1 μg/mg of protein), 10 mM-KH_2PO_4, 5 mM-succinate and Ca^{2+} (50 nmol/mg of protein). Under these conditions, Ca^{2+} accumulation by the mitochondria leads to maximal permeabilization within 10 min (assayed as described by Crompton & Costi, 1988).

The measurements were made with a rapid-mixing pulsed-flow device competent to measure changes occurring in 50 ms (Crompton & Costi, 1988). The procedure is represented schematically as follows, where 'Solute' refers to the solute whose entrapment is measured:



The various media all contained 120 mM-KCl, 10 mM-Hepes, 10 mM-KH_2PO_4 and 5 mM-succinate. Further additions were as follows: in the 'solute'-containing medium, either [$U^{14}C$]mannitol, [U-^{14}C]sucrose or arsenazo III (all at 5 mM) and ^3H_2O; in the resealing medium, 10 mM-EGTA and, in order to prevent their dilution, ^3H_2O and 'solute'; in the dilution medium, 10 mM-EGTA. In essence, mitochondria permeabilized as described above were mixed with the 'solute' investigated, which entered the mitochondria during time period t_1. Addition of resealing medium containing 10 mM-EGTA to chelate Ca^{2+} induced pore closure during the time interval t_2. The mixture was then diluted with a 5-fold volume of dilution medium and the mitochondria were sedimented 1 min (t_3) later. The degree of 'solute' entrapment in the matrix space was calculated from the ^3H_2O and 'solute' concentrations of the supernatants and the mitochondrial pellets. Arsenazo III was determined spectrophotometrically at 690−650 nm in the presence of 300 μM-Ca^{2+}.

The principle of the method is that 'solute' that has entered the permeabilized mitochondria during t_1 and that has become entrapped owing to pore closure during t_2 would not be diluted from the matrix space on dilution. Thus solute entrapment would depend on the following rate constants: k_p^*, the apparent rate constant for 'solute' permeation (entry) in the presence of Ca^{2+} during t_1; k_r, the apparent rate constant for mitochondrial resealing during t_2 and t_3; and k_p, the apparent rate constant for 'solute' permeation in the absence of Ca^{2+}, i.e. during resealing; it is perhaps important to clarify that although the permeability of the mitochondrial population as a whole obviously decreases with time as mitochondria become resealed, k_p refers to the permeability of that fraction of the population that is unresealed at any point in time during resealing.

Solute entrapments at time t (either t_1 or t_2) and at infinite time are expressed by the terms S_t and S_∞ respectively.

Mitochondrial resealing with time. The rate of resealing was measured by prolonging t_1 to allow complete 'solute' equilibration during this time and by varying t_2. Entrapment data are consistent with pores of any single mitochondrion closing simultaneously on Ca^{2+} removal (Crompton & Costi, 1988), which is referred to as 'mitochondrial resealing'. Accordingly, exponential resealing of mitochondria with time leads to the expression (eqn. 3 in Crompton & Costi, 1988):

$$S_t = S_\infty[1-e^{-k_r t_2}(1-e^{-k_p/k_r})]$$

which rearranges to the linear form:

$$\ln\left[1-\frac{S_t}{S_\infty}\right] = -k_r t_2 + \ln[1-e^{-k_p/k_r}] \quad (1)$$

Plots of $\ln[1-S_t/S_\infty]$ versus t_2 yield k_r (slope) from which k_p, the apparent rate constant for permeation during the period of resealing in t_3, may be derived (intercept).

Solute permeation with time. This was measured by varying t_1 and by prolonging t_2 to allow complete mitochondrial resealing before dilution. The same assumptions as in resealing (above) lead to the expression (eqn. 6 in Crompton & Costi, 1988):

$$S_t = S_\infty[1-e^{-k_p^* t_1}\cdot e^{-k_p/k_r}]$$

which converts into the linear form:

$$\ln\left[1-\frac{S_t}{S_\infty}\right] = -k_p^* t_1 - \frac{k_p}{k_r} \quad (2)$$

Plots of $\ln[1-S_t/S_\infty]$ versus t_1 yield k_p^* (slope). If k_r is known, one may derive k_p, the apparent rate constant for permeation during the period of resealing in t_2 (intercept).

Analysis of permeation data. Rates of permeation (P) were analysed in terms of permeation via a cylindrical pore according to the Renkin (1954) equation

$$P \propto D[1-(r_s/r_p)]^2[1-2.11(r_s/r_p) + 2.09(r_s/r_p)^3 - 0.95(r_s/r_p)^5]$$

where r_s and r_p are the radii of the permeating solute and the pore respectively. D is the diffusion coefficient of the solute in aqueous solution. Relative values of D were calculated on the assumption that, for small molecules, $D \propto M_r^{-0.5}$ (Stein, 1967). The approximate dimensions of the solutes investigated were measured from Drieding models. The dimensions used were the transverse van der Waals radii as follows: mannitol, 0.29 nm; sucrose, 0.4 nm; arsenazo III, 0.75 nm (with one water molecule associated with each acid group).

RESULTS AND DISCUSSION

Analysis of solute-entrapment data

Measurements of solute entry by solute entrapment are complicated by the fact that resealing (used to stop the process) is not instantaneous, so that account must be taken of the rate of resealing and the additional solute entry that occurs during the resealing phase. Similarly,

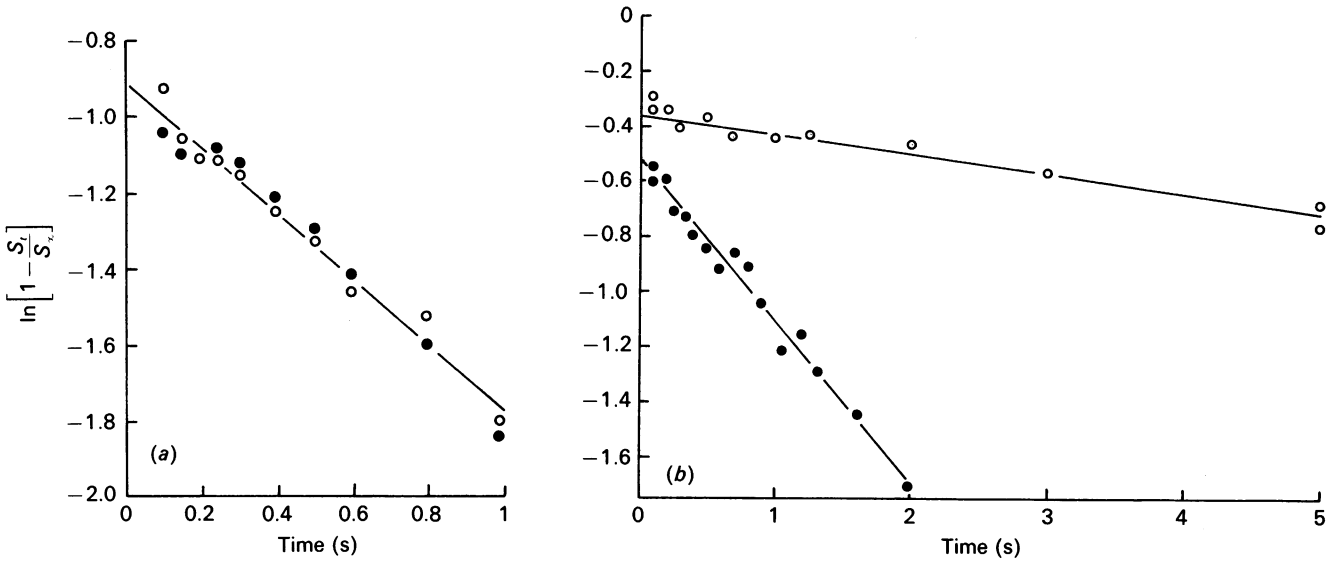


Fig. 1. Determination of the apparent rate constants for sucrose permeation during (*b*) and in the absence of (*a*) net resealing

(*a*) Sucrose entrapments were determined in the presence (●) or absence (○) of 200 μM-ADP as the time period t_1 (the Methods section) was varied from 100 ms to 1 s, and the data were plotted according to eqn. (2) in the Methods section. The mitochondria were resealed with EGTA plus 200 μM-ADP. (*b*) Sucrose entrapments were measured as the resealing time period t_2 (see the Methods section) was varied from 100 ms to 5 s, and the data were plotted according to eqn. (1) (see the Methods section). The mitochondria were resealed with EGTA alone (○) or with EGTA plus 200 μM-ADP (●).

during resealing-rate measurements, solute loss from the non-resealed fraction on dilution (used to stop entrapment during resealing) is not instantaneous, so that one must take account of the rate of permeation during the completion of resealing after dilution. These factors are incorporated into eqns. (1) and (2), both of which are based on two conditions (Crompton & Costi, 1988): (*a*) that the net rate of solute permeation is directly proportional to the difference in matrix and external [solute], and (*b*) that pores in any mitochondrion close 'simultaneously' (resealing) and mitochondria reseal exponentially with time. These conditions may be assessed empirically from the extent to which the predicted linearity between $\ln[1-S_t/S_\infty]$ and either the permeation period t_1 (condition *a*) or the resealing period t_2 (condition *b*) is actually observed.

Fig. 1(*a*) tests condition (*a*). A linear relationship was obtained over the range 100 ms–1 s; net permeation was about 85% complete at 1 s, and measurements with longer permeation times became inconsistent. Fig. 1(*b*) tests condition (*b*), and again a linear relation was obtained. It may be concluded that both conditions hold and that the analyses may be applied with some confidence.

Fig. 1(*b*) shows that the rate of resealing of permeabilized mitochondria on addition of EGTA was increased 10-fold by ADP (negative slope = k_r). Similar experiments (not shown) revealed little or no effect of ATP (0.5 mM) on the rate of EGTA-induced resealing, in agreement with previous conclusions that EGTA-induced resealing is stimulated specifically by ADP (Crompton & Costi, 1988). The permeabilities of the unresealed fraction at any point in time during resealing (k_p) were calculated from the values of the ordinate intercepts and k_r. The values of k_p (sucrose) thus determined were 0.07 s^{-1} (minus ADP) and 0.52 s^{-1} (plus ADP), as reported in Table 1.

The permeabilities in the presence of Ca^{2+} (i.e. without resealing) were obtained from Fig. 1(*a*) (negative slope = k_p^*). The plot yields a value for k_p^* (sucrose) of 0.84 s^{-1} in both the presence and absence of ADP. The ordinate intercept of Fig. 1(*a*) allows calculation of k_p (plus ADP, present in the resealing medium) if k_r is known. Since the value of k_r (plus ADP) was 0.58 s^{-1} (from Fig. 1*b*; see

Table 1. Effect of ADP on the apparent rate constants for sucrose permeation and for resealing

The values were obtained from Fig. 1 and from similar experiments, as indicated, in the presence and absence of 200 μM-ADP.

Expt.	$k_r(s^{-1})$		$k_p(s^{-1})$		$k_p^*(s^{-1})$
	−ADP	+ADP	−ADP	+ADP	±ADP
Fig. 1(*a*)	–	–	–	0.53†	0.84
Fig. 1(*b*)	0.06	0.58	0.07	0.52	–
Four expts. (mean ± S.E.M.)	0.07±0.02	0.75±0.10	0.09±0.02	0.64±0.15	0.92±0.17

† Value obtained by using the k_r value from Fig. 1(*b*).

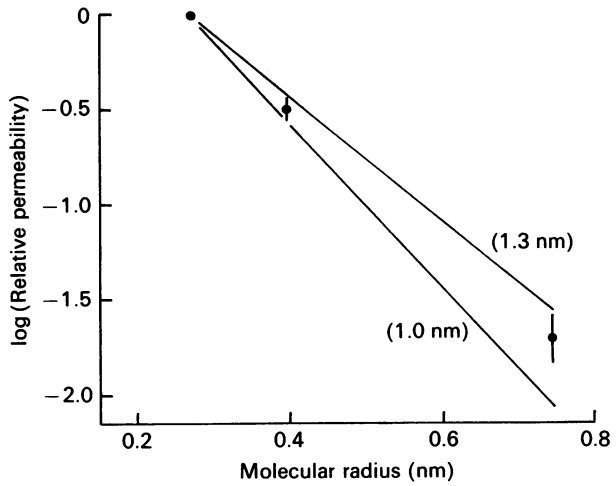


Fig. 2. Relationship between the apparent rate constants for solute permeation and molecular radii

The values of k_p (means ± S.E.M.; three determinations) for mannitol, sucrose and arsenazo III were determined as in Fig. 1(*b*) and expressed relative to that of mannitol. The continuous lines are theoretical curves drawn according to the Renkin equation (see the Methods section) for pore radii of 1.0 nm and 1.3 nm (given in parentheses).

Table 1), a k_p (sucrose) value of 0.53 s^{-1} is indicated, in agreement with that obtained from Fig. 1(*b*) (0.52 s^{-1}; Table 1).

In summary, the plotting procedures appear to provide reliable means for the determination of basic parameters by the pulsed-flow solute-entrapment technique. At present, however, influx measurements are constrained by the rate of mitochondrial resealing, and even the highest rate of resealing obtained with EGTA plus ADP was insufficiently high to yield time courses of entry for solutes permeating more rapidly than sucrose. Therefore, the permeation of different solutes (below) was compared by means of their k_p values obtained from plots of the type shown in Fig. 1(*b*).

Effect of ADP on the relative permeability to different solutes

It is notable that ADP stimulated not only resealing (k_r) but also the permeability (k_p) of the unresealed fraction during the resealing phase. On the other hand, the permeability (k_p^*) in the absence of net resealing was unaffected. As reported in Table 1, in four such experiments, ADP increased k_p about 7-fold, to a value approaching k_p^*. As derived, k_p comprises both the number of open pores in unresealed mitochondria and the permeabilities of individual pores and, in principle, ADP may increase k_p by increasing either of these parameters. In order to discriminate between these possibilities, an indication was obtained of pore size and how this would need to change if changes in individual pore permeability were the cause.

Fig. 2 reports relative k_p values for three solutes of different size (M_r 180–780) obtained in the presence of ADP. As expected, there was an inverse relation between permeability and molecular size, mannitol permeating about 3-fold faster than sucrose and about 40-fold faster than arsenazo III. The Renkin equation (see the Methods section) has been applied in many studies of permeation via pores (Ginsburg & Stein, 1987 and references cited therein). The equation is derived from consideration of the probability of the solute entering the pore and the frictional drag between pore wall and solute. Fig. 2 shows theoretical lines according to the Renkin equation from which it is seen that the experimental data are consistent with permeation through pores of radii 1.0–1.3 nm.

In all probability, putative pore radii obtained in this way represent an approximation at best (discussed by Ginsburg & Stein, 1987). Nevertheless, the indication that molecules of radius greater than about 1.3 nm would be impermeant is consistent with earlier observations of the osmotic behaviour of Ca^{2+}-treated mitochondria suspended in poly(ethylene glycol) species with a range of M_r values and which suggested a sharp cut-off in permeability at M_r 1500 (Haworth & Hunter, 1980). Thus the hydrodynamic radius of poly(ethylene glycol) 1500 is given as about 1.2 nm (Ginsburg & Stein, 1987), although clearly the 'effective' radius for permeation of long linear molecules is not known.

If an effective pore radius of 1.15 nm is assumed, and ADP is considered to affect pore radius, then the Renkin equation predicts that a decrease in radius to about 0.69 nm would be needed to account for the 7-fold decrease in k_p (sucrose) in the absence of ADP. According to this, arsenazo III (radius 0.75 nm) would not be expected to permeate at all in the absence of ADP. In view of uncertainties, such precise predictions are probably unwarranted; nevertheless, if ADP affected pore size, then permeation of the larger molecular arsenazo III would certainly be limited more by the absence of ADP than permeation of sucrose. Yet, as shown in Fig. 3, the k_p values for sucrose and arsenazo III were affected equally by ADP, the ratio k_p (sucrose)/k_p (arsenazo III) being maintained at a value of about 12 in spite of the many-fold change in permeation rates. This would seem to eliminate changes in pore size as the reason for the ADP-induced increase in k_p and to suggest, instead, that an increase in open-pore number was the cause.

Resealing and the rate of matrix Ca^{2+} removal

At first sight it seems quite paradoxical that ADP should increase both the rate of resealing and the number of open pores in the unresealed fraction at any point in time during resealing; certainly, it would be difficult to account for in terms of the most obvious sequence for resealing:

$$\underset{\text{permeable}}{\text{Most}} \longrightarrow \underset{\text{permeable}}{\text{least}} \rightarrow \underset{\text{(resealed)}}{\text{impermeable}}$$

Whereas the effect of ADP on k_r indicates displacement to the right, that on k_p suggests displacement to the left.

As discussed previously (Crompton & Costi, 1988), solute entrapment during resealing is consistent with all pores of any single mitochondrion closing 'simultaneously' on Ca^{2+} removal. Taken together with the present data (discussed below), this suggests the sequence:

$$\underset{\text{permeable}}{\text{Least}} \overset{\text{ADP}}{\underset{\text{Slow}}{\longleftarrow\!\!\!\longrightarrow}} \underset{\text{permeable}}{\text{most}} \overset{\text{Fast}}{\longrightarrow} \underset{\text{(resealed)}}{\text{impermeable}}$$

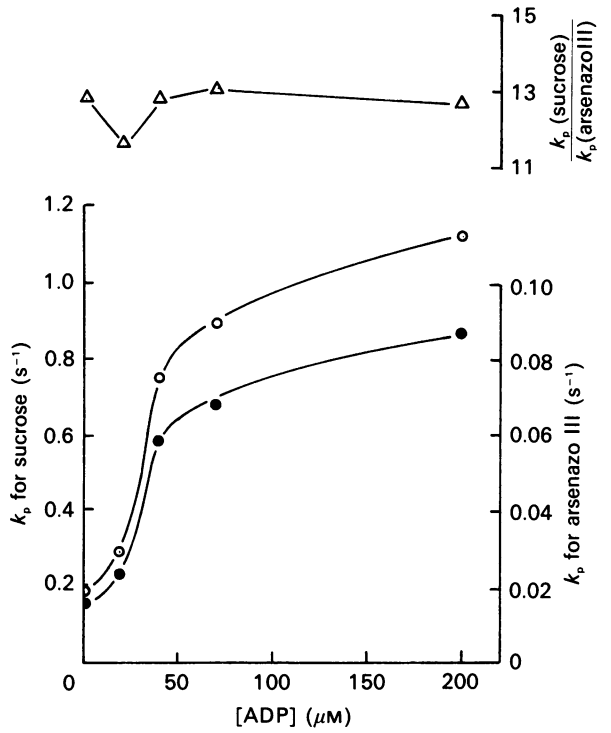


Fig. 3. Dependence of the apparent rate constants of sucrose and arsenazo III permeation on [ADP]

The values of k_p for [^{14}C]sucrose (○) and arsenazo III (●) were determined by the method described in Fig. 1(*b*). The ratios between the k_p values of the two solutes (△) are given in the upper panel.

A possible cause might be that the rate of removal of free Ca^{2+} from the matrix space is limiting for EGTA-induced resealing and that pore closure *per se* (in Ca^{2+}-free mitochondria) is relatively fast. This is not unreasonable, since a pore radius of around 1.1 nm as indicated would be predicted to restrict movement of both Ca^{2+} and EGTA. From Dreiding models, we determined a minimal molecular radius of EGTA (uncomplexed to metals) of about 0.37 nm, so that permeability to EGTA may not differ greatly from that of sucrose. Ca^{2+} is heavily hydrated, and one may calculate a hydrated-cation radius of about 0.42 nm [as described by Robinson & Stokes (1959) with ten water molecules/cation]. Associated Cl^- (crystallographic radius 0.18 nm) would increase the dimension by about 0.23 nm, assuming that the anion penetrates by 0.13 nm into the hydration shell (Robinson & Stokes, 1959). Thus if, as seems likely, the permeant species is $CaCl_2$, one might expect a permeability about 8-fold lower than for sucrose (from Fig. 2). In addition, EGTA was added in 200-fold excess over Ca^{2+} on resealing, and only a small part of the total EGTA flux across the inner membrane would be necessary for complete chelation of matrix Ca^{2+}. In all probability, therefore, resealing was caused by entry of EGTA and chelation of matrix Ca^{2+} rather than by Ca^{2+} efflux.

According to the hypothesis, mitochondria with the greatest number of open pores would be resealed fastest by EGTA, since they would be depleted of free Ca^{2+} most quickly. Consequently, EGTA would selectively reseal the most permeable forms, and the distribution of unresealed mitochondria between least and most permeable would be shifted to the least permeable on net resealing. In agreement with this, the permeability rate constant was markedly decreased on net resealing in the absence of ADP (i.e. $k_p < k_p^*$; Table 1). If ADP facilitated the interconversion of pores between the open and closed states (above), then ADP would be expected to increase the proportion of unresealed mitochondria in the most permeable states during net resealing, and both k_r and k_p would be increased, as observed. In fact, as shown in Fig. 4, there was a linear relation between these two parameters as [ADP] was increased, in agreement with the hypothesis. On the other hand, we observed no detectable effect of ADP on mitochondrial permeability in the absence of net resealing (k_p^*; Fig. 1*a*), which suggests that ADP did not shift the equilibrium distribution between open and closed forms in the presence of Ca^{2+}. It may be that ADP facilitates both pore opening and closure in permeabilized mitochondria, or that ADP was simply ineffective in the presence of high [Ca^{2+}].

The hypothesis rests on the assumption that removal of free Ca^{2+} from the matrix space by chelation with EGTA within the matrix is rate-limiting for EGTA-induced resealing. This was tested by investigating the effect on resealing of the Ca^{2+} ionophore A23187, which would be expected to increase the rate of matrix Ca^{2+} removal on addition of EGTA. A23187 mediates $2H^+/Ca^{2+}$ exchange, but rapid H^+ permeation via the pore (Al Nasser & Crompton, 1986*a*) would be expected to prevent matrix acidification on Ca^{2+} efflux. As reported in Fig. 5, A23187 caused a 3-fold increase in k_r, in agreement with the premise. The ionophore also caused a smaller (50%) increase in k_p; this would be predicted since, in the presence of ionophore, net resealing would discriminate less against the least-permeable forms.

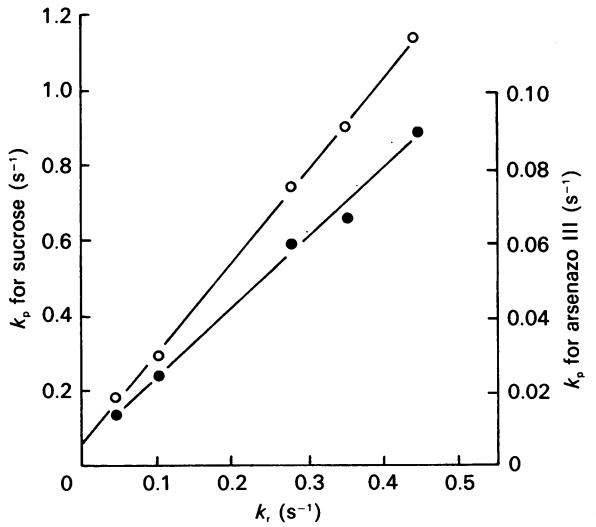


Fig. 4. Relationship between the apparent rate constants for solute permeation and for resealing

The values of k_p for sucrose (○) and arsenazo III (●) and the values of k_r were obtained by the method of Fig. 1(*b*) as [ADP] was varied from 0 to 200 μM.

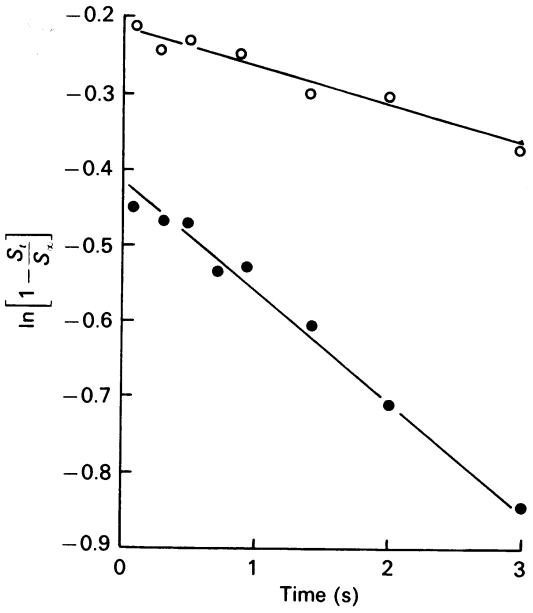


Fig. 5. Effect of Ca^{2+} ionophore on the rate of EGTA-induced resealing

Sucrose entrapments were measured as the time period t_2 (see the Methods section) was varied from 100 ms to 3 s. The data are plotted according to eqn. (1) in the Methods section. The mitochondria were resealed with EGTA alone (○) or with EGTA plus A23187 (5 nmol/mg of protein) (●). The plots yield the following values (s^{-1}): (*a*) minus A23187: $k_r = 0.5$; $k_p = 1.1$; (*b*) plus A23187: $k_r = 1.5$; $k_p = 1.6$.

Conclusions

The relative permeability to different solutes is consistent with permeation via a pore of about 2.3 nm diameter, although it does not exclude leak pathways arising from Ca^{2+}-activated phospholipase A_2 action, since leaks could also discriminate on the basis of molecular size. Our proposed involvement of a discrete protein pore is really founded on two conditions.

Firstly, mitochondria permeabilized as they were in the present study are completely uncoupled and devoid of ATP, and resealing without energy input cannot be attributed to membrane repair involving phospholipid reacylation (Al Nasser & Crompton, 1986*a*; Crompton & Costi, 1988). This point is emphasized by the implication of the present study that the true rate of resealing of the most permeable forms occurs much faster than is apparent from resealing of the whole population. An indication of the possible rate of pore closure in the most permeable forms may be obtained. Thus we observed that, on addition of EGTA in the absence of ADP, there occurred a 10-fold decrease in the rate constant for permeation, i.e. from 0.92 s^{-1} to 0.09 s^{-1} (k_p^* and k_p; Table 1). Since the plots used to derive these values were linear to <200 ms (e.g. Fig. 1), the change evidently occurred within this time; this is equivalent to a first-order rate constant >11 s^{-1} ($t_{\frac{1}{2}} < 60$ ms). We suggest that this rapid decrease in permeability may reflect resealing of the most permeable forms and that pore closure *per se* (in Ca^{2+}-free mitochondria) may occur within a half-time of 60 ms. Further investigation of this awaits development of appropriate assay procedures.

Secondly, the pore concept implies an equilibrium between open and closed states of the pore in permeabilized mitochondria. It is not easy to design experiments to test this, although we have shown previously that, at intermediate concentrations of matrix free Ca^{2+}, when permeabilization is far from maximal, labelled sucrose eventually enters the whole population despite maintainance of the inner-membrane potential at a constant value with time, which is consistent with a dynamic distribution between closed and open states (Al Nasser & Crompton, 1986*a*). In this respect, our finding that the rate of removal of matrix free Ca^{2+} is limiting for resealing is fortuitous, because, in effect, it permits discrimination between mitochondria of differing permeabilities. Since ADP markedly increased permeability, without apparently changing the dimensions of the permeability pathway, an increase in the number of such pathways is indicated, and we have suggested how this might occur in terms of an altered pore-state distribution. An analogous situation may be envisaged with leak pathways, which may be transient in nature, but it is not easy to see how this might be influenced specifically by low (physiological) concentrations of ADP. These effects are more readily interpreted in terms of ADP binding to a protein, conceivably the pore itself, and by its facilitating the interconversion between the open and closed states, without change in pore size. We have also reported that pore opening is blocked by low concentrations of the immunosuppressant cyclosporin, although it is not yet known whether cyclosporin binds to the pore itself or to a protein involved in some way in pore opening (Crompton *et al.*, 1988).

In conclusion, the seemingly paradoxical effects of ADP exposed in the present study are quite consistent with previous indications of a reversible Ca^{2+}-activated pore (Al Nasser & Crompton, 1986*a*; Crompton & Costi, 1988). Although its physiological role is obscure, the pore may well be the locus of mitochondrial dysfunction during tissue Ca^{2+} overload (see the Introduction) and offer a suitable target for pharmacological interventions designed to prevent or retard the onset of irreversible tissue injury.

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